Study of mycorrhizae by means of fluorescent antibody[1]

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The fluorescent antibody technique was found to be highly promising as a new approach to the study of mycorrhizae. Fluorescent antibodies prepared against two mycorrhizal fungi, *Thelephora terrestris* and *Pisolithus tinctorius*, were effective and specific as microscopic stains for those fungi and their corresponding mycorrhizae. *Thelephora terrestris* antibody gave zero, and *P. tinctorius* antibody gave five cross-reactions of moderate intensity, in tests with 31 diverse nonmycorrhizal fungi. *Armillaria mellea* reacted nonspecifically with several fluorescent antibodies.

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On a obtenu du succès en utilisant la technique d'immunofluorescence pour l'étude des mycorrhizes. On a preparé des anticorps fluorescents pour deux champignons mycorrhiziens, *Thelephora terrestris* et *Pisolithus tinctorius*. Ces anticorps ont été utilisés avec succès en tant que colorants microscopiques spécifiques à ces champignons et aux mycorrhizes correspondantes. Au cours d'essais portant sur 31 champignons non mycorrhiziens, l'anticorps de *T. terrestris* n'a presenté aucune réaction croisée; l'anticorps de *P. tinctorius* a presenté cinq réactions croisées de faible intensité. *Armillaria mellea* a presenté des réactions non spécifiques avec plusieurs anticorps fluorescents.

The composite organ, termed mycorrhiza, that results from the association of certain soil fungi with the roots of selected higher plant partners, is generally conceded to be extremely widespread and of great biological significance (3, 4). A vast and controversial literature attests not only to the importance of mycorrhizae but as well to the difficulties inherent in their study. As noted by Zak (9), so basic a capability as that of identifying the fungus involved in a natural mycorrhizal structure is sharply limited by existing techniques.

We have explored the use of the fluorescent antibody (FA) technique as a new approach to the study of mycorrhizae and find it to be of substantial promise for the identification and characterization of mycorrhizal associations in nature, and for general ecological study of mycorrhizal fungi. The FA approach is particularly attractive for such problems in microbial autecology as it offers the only possibility to simultaneously observe and identify a morphologically nondescript microorganism in its complex natural environment. The technique and experience relative to other applications in microbial ecology were reviewed recently (7).

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Cultures of the mycorrhizal fungi *Pisolithus tinctorius* and *Thelephora terrestris* were used individually as antigens. Each was grown in modified Melin-Norkrans medium (5) for 10–14 days, washed, homogenized, and diluted to 0.5 mg/ml in sterile saline. Suspensions were injected into rabbits according to the following schedule: days 1, 3, and 5—0.5, 1.0, and 1.5 respectively, intravenous (i.v.); days 5–9—no injection; days 9 and 11—1.5 and 2.0 ml respectively, i.v.; days 11–15—no injection; days 15 and 19—2.0 ml subcutaneous (s.c.); rest 1 week then collect blood by cardiac puncture. Agglutination titers exceeded 1280. Procedures for antiserum fractionation, labeling with fluorescein isothiocyanate (FITC), staining with the resultant fluorescent antibody, examination by fluorescence microscopy, and photomicrography were as reported previously (2, 8).

Successful immunofluorescence examination requires at the outset that the FA be active in reacting with its antigen (the homologous system). Each mycorrhizal fungus FA as first tested on mycelial smears reacted strongly with its corresponding fungus antigen. Hyphae of *P. tinctorius* stained with *P. tinctorius*-FA (Pt-FA) appeared under the fluorescence microscope with

the bright apple-green fluorescence characteristic of the FITC, as did hyphae of *T. terrestris* stained with *T. terrestris*-FA (Tt-FA). In both homologous systems the fluorescence was uniform and rated 4+ to indicate maximum intensity. An undesirable degree of cross-reaction was noted in that Pt-FA reacted with *T. terrestris* at the moderate two to three intensity level; Tt-FA, although apparently more specific, did react weakly (1+ to 2) with *P. tinctorius*. Cross-reactions were due to common antigens and not to nonspecific staining since a bacterial FA (to *Rhizobium japonicum*, A24) did not react with either fungus.

Each mycorrhizal fungus FA was adsorbed to increase specificity. The adsorption procedure for Pt-FA was typical: 200 mg of *T. terrestris* homogenate was centrifuged and 5 ml of Pt-FA was mixed into the pellet. After 1.5 h incubation at 4C the mycelium was removed by centrifugation. The procedure was repeated and the final supernatant Pt-FA adsorbed with Tt(Pt/ads Tt) was passed through Whatman No. 5 filter to remove fungal debris. As seen in Fig. 1*a* and *b*, adsorptions did not result in material reduction of the activity of either FA in its homologous system.

Additional tests to examine the specificity of the two adsorbed fungal FA preparations are summarized in Table 1. The *T. terrestris* FA was especially satisfactory in that it reacted strongly with the mycelium of all *T. terrestris* isolates and essentially negatively with all other fungi tested. A single exception was the *Armillaria mellea* culture which reacted nonspecifically with Tt-FA, Pt-FA, and with each of several bacterial FA preparations. We have not encountered such nonspecific staining in prior work, and do not know why the hyphae of *A. mellea* are stained strongly by any FA. One hypothesis is that the surfaces of *A. mellea* may be spongy or porous or otherwise prone to trap any added FA. Occasionally spores or sporangia gave a strongly positive FA reaction even though associated with entirely negatively staining mycelia. The indiscriminate FA staining of certain fungus spores has been seen before,[2] and was encountered in this study particularly in the genus *Peniophora*. *Peniophora tinctorius* FA demonstrated somewhat less specificity than the Tt-FA, in that some interfering staining occurred on hyphae of seven unrelated fungi. Of these, the five that showed most intensive cross-reactions (2+ to 3 level) were *Corticium olivascens*, *Peniophora cinerea*, *P. sanguinea*, *Veluticeps berkeleyi*, and *Lepista nuda*.

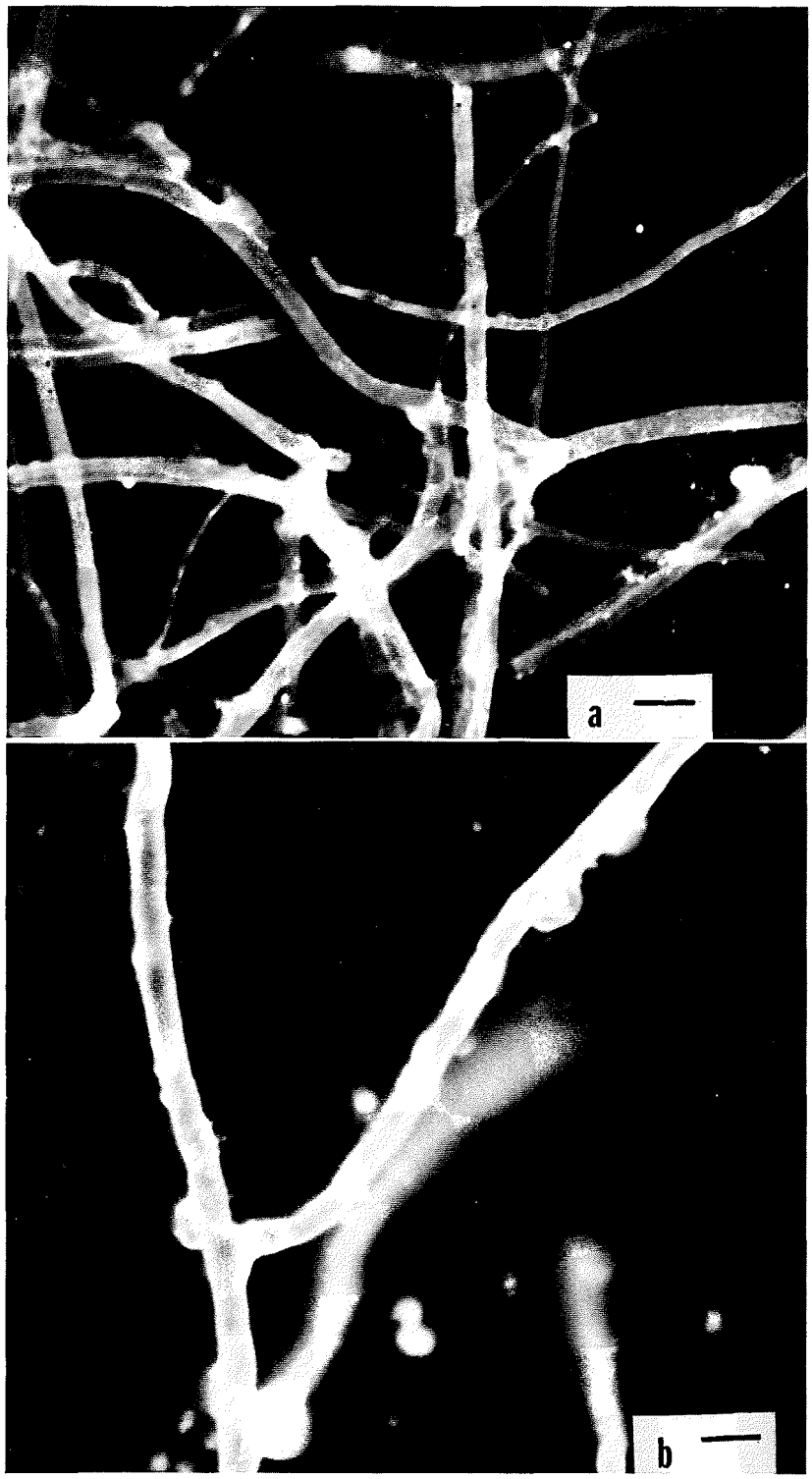
That both mycorrhizal fungus FA preparations are useful and specific for staining mycorrhizae is shown in Fig. 1*c* and *d*. Figure 1*c* is a photomicrograph of a cultured (6) *P. tinctorius* ectomycorrhiza of red pine, prepared from a razor blade section, and stained with Pt-FA. The Hartig net is seen clearly as distinct interconnected white lines; these appeared under the microscope as bright 4+ specific apple-green fluorescence. The same mycorrhiza stained with Tt-FA or a bacterial FA gave no evidence of specific fluorescence and appeared orange-brown due to the gelatin rhodamine counterstain (1) used on all preparations. Although razor blade sections can be satisfactory, the thickness of such preparations leads to background fluorescence from underlying tissues, and this accounts for the diffuse light zone surrounding the tissue in focus in Fig. 1*c*. However, tissue thickness is more troublesome for photomicrography than for microscopic examination where the focus can change.

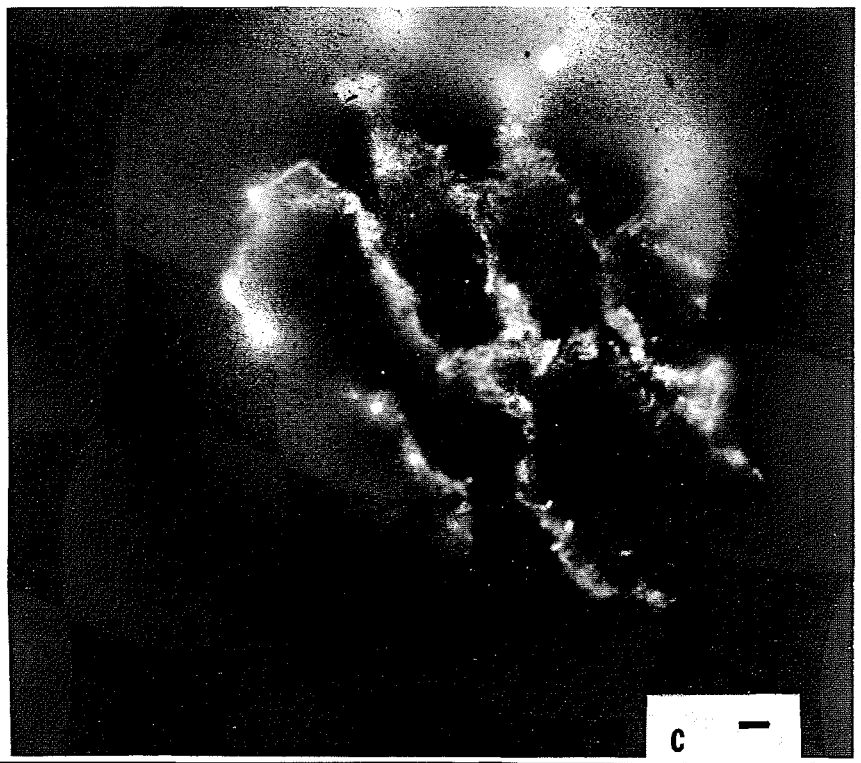
Microtome sections of 4–6 micrometers (μm) were highly satisfactory for FA examination. One such microtome section shown in Fig. 1*d* demonstrates specific Tt-FA staining of the Hartig net and mantle of a cultured ectomy-

[2]Nearly all problems of nonspecific binding of FA to surfaces in natural environments are controlled by routine use of gelatin-rhodamine treatment (1); however, we have found conidia of a few aspergilli and those of a *Fusidium* that apparently trap and retain FA nonspecifically, despite this treatment.

FIG. 1. Immunofluorescent microscopic fields of mycorrhizal fungi and ectomycorrhizae of pine. (*a*) Contact slide preparation of *T. terrestris* stained with Tt/ads Pt-FA. (*b*) Contact slide of *P. tinctorius* stained with Pt/ads Tt-FA. (*c*) Razor blade section of the Hartig net of *P. tinctorius* mycorrhiza stained with Pt/ads Tt-FA. (*d*) Freezing microtome section of Tt mycorrhiza stained with Tt/ads Pt-FA. Bright filaments of mycelia (*a*, *b*) Hartig net (*c*, *d*) and mantle (*d*) fluoresced with 3–4+ specific yellow-green color when viewed under the microscope. In (*d*) bright circular portion interior to Hartig net fluoresced orange-brown due to counter stain taken up by plant tissue. The three circular structures seen in the cortical cells are artifacts due to entrapment of air. Magnification lines represent 10 μm.

PLATE I

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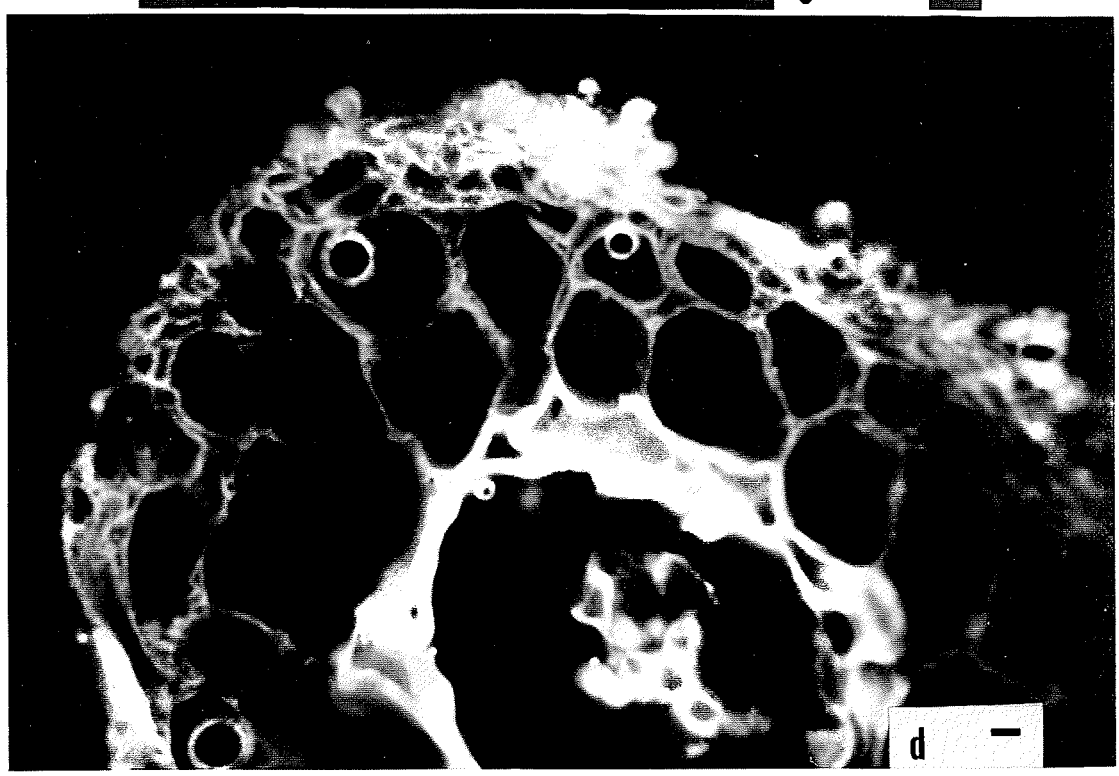
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TABLE 1

Immunofluorescence reactions of a range of fungi stained with fluorescent antibodies of the mycorrhizal fungi *T. terrestris* and *P. tinctorius*

Isolates*	FA staining reaction†	
	Tt/ads Pt	Pt/ads Tt
Mycorrhizal		
Pisolithus tinctorius 49(55)	±	4+
P. tinctorius 49(58)	±	4+
Thelephora terrestris Z-13	4+	±
T. terrestris 54	3–4	±
T. terrestris Z-65	4+	±
T. terrestris V-9037	4+	±
T. terrestris MST-12	3–4	1
T. terrestris MST-14	4+	1
Lepista nuda Cr-180(21)	±	2–3
Armillaria mellea P-14	NSS	NSS
Laccaria laccata (15)	±	1
Nonmycorrhizal Thelephoraceae		
Coniophora arida	±	±
Corticium galactinum	±	±
C. olivascens	±	2
Gloeocystidiellum porosum	±	±
Hymenochaete rubiginosa	±	±
H. tabacina	±	±
Laxitextum crassum	±	1–2
Peniophora gigantea	±	±
P. cinerea	Neg	3
P. flavido-alba	Neg	±
P. sanguinea	Neg	2
Sterum complicatum	±	±
S. gansapatum	Neg	±
S. sanguinolentum	±	±
S. subpileatum	±	±
Veluticeps berkeleyi	1	3
Other fungi		
Pythium aphanidermatum	Neg	Neg
P. vexans	Neg	Neg
Pytophthora cactorum	Neg	Neg
P. cinnamomi	Neg	Neg
P. hevea	Neg	Neg
P. parasitica	Neg	Neg
Chaetomium globosum	Neg	NT
Gymnoascus sp.	Neg	Neg
Sordaria fimicola	Neg	±
Ctenomyces serratus	±	1
Fusarium solani	Neg	Neg
Penicillium sp.	Neg	Neg
Sporobolomyces roseum	Neg	NT
Agaricus bisporus	±	2
Pleurotus ostreatus	±	NT

*Isolates were grown in pure culture on contact slides buried in sand : vermiculite : perlite (1:1:1) moistened with Melin-Norkrans medium (5). Numbers in parenthesis are D. H. Marx stock culture designation.

†Staining reaction symbols: NT, not tested; neg, no fluorescence; ±, trace; 1+ to 4+, increasingly good specific fluorescence; NSS, nonspecific staining. Tt/ads Pt is *T. terrestris* FA absorbed with *P. tinctorius* mycelium and Pt/ads Tt is *P. tinctorius* FA adsorbed with *T. terrestris* mycelium. All FA preparations were used at 1:4 dilution.

corrhiza of *T. terrestris* of Virginia pine. The specific 3–4+ fluorescence, appearing in this photomicrograph as delineated white areas, was found only when the *T. terrestris* mycorrhiza sections were stained with Tt-FA. Microtome sections of *P. tinctorius* ectomycorrhizae were equally definitive in FA reaction and equally negative with respect to non-homologous FA controls. Autofluorescence was not a problem in any of the plant or fungus tissues examined.

The success of these initial studies with *P. tinctorius* and *T. terrestris* points to the fluorescent antibody as a new and effective approach to problems that have been notoriously refractory in mycorrhiza research. The avenues thus exposed for significant contributions by the fluorescent antibody approach include the rapid identification of mycorrhizal fungi on various plant partners; appraisal of the free-living behavior and vegetative activity of mycorrhizal fungi in soil apart from the host; and opportunity to circumvent some of the problems in aseptic synthesis of mycorrhizal structures and those involved in the culture of fastidious fungi presumed to be involved in many mycorrhizae.

Acknowledgments

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